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B cell Receptor Signaling in Diffuse Large B cell Lymphoma

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Abstract

The importance of understanding the genetic and biochemical basis of B cell receptor (BCR) survival signaling in diffuse large B cell lymphoma (DLBCL) is underscored by the recent clinical success of agents that target the BCR pathway. DLBCL is composed of multiple distinct molecular subtypes with divergent clinical outcomes. The activated B cell-like (ABC) subtype is the most aggressive form of DLBCL and is often resistant to standard chemotherapies. ABC DLBCL expresses numerous genes found in antigen-activated B cells, and genetic and pharmacologic studies have demonstrated that ABC DLBCL tumors are addicted to NF- κ B activity. The origins of this NF- κ B activity remained obscure until RNA interference screens established that the majority of ABC DLBCL cell lines rely on expression of BCR components and downstream signaling effectors for NF- κ B activation. Pharmacological inhibition with ibrutinib of Bruton's tyrosine kinase (Btk), a kinase that is required for BCR signaling to engage NF- κ B, is selectively toxic for ABC DLBCL tumors; a finding that has now been translated to the clinic. These novel targets not only offer a promising new therapy options for ABC DLBCL, but also demonstrate the value of a deep molecular understanding of oncogenic signaling pathways.

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common lymphoid malignancy representing more than 20,000 new cases per year (<http://seer.cancer.gov/statfacts/html/leuks.html>). Although DLBCL is commonly described as one disease, gene expression profiling has identified distinct molecular subtypes. The activated B cell-like (ABC) and germinal center B cell-like (GCB) subtypes make up the majority of DLBCL cases and are found at roughly equal frequency [1, 2]. GCB DLBCL tumors express many genes found in normal germinal center B cells, while gene expression in ABC DLBCL resembles that of antigen-activated B cells. In particular, ABC DLBCL expresses many targets of NF- κ B signaling [3]. ABC DLBCL has an inferior clinical outcome compared to GCB DLBCL,

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with an overall survival of ~40% [4]. The inability of chemotherapy to eradicate the majority of ABC DLBCL tumors may be due to their constitutive activation of the NF- κ B pathway, which is a potent anti-apoptotic mechanism that can blunt the activity of cytotoxic agents [5].

Oncogenic NF- κ B signaling in ABC DLBCL

ABC DLBCL lines express NF- κ B-dependent genes and are addicted to NF- κ B activation for their survival. The term “NF- κ B” signifies a family of hetero- and homo-dimeric transcriptional activators that are sequestered in the cytoplasm of resting cells, rendering them inert [6]. Signals from various activation stimuli converge on I κ B kinase (IKK), which phosphorylates the cytoplasmic inhibitor of NF- κ B, I κ B α , resulting in its degradation. This releases the NF- κ B factors to translocate to the nucleus, bind DNA, recruit cofactors and activate transcription of many target genes. NF- κ B activity in ABC DLBCL is due to constitutive activation of IKK β , which in turn phosphorylates I κ B α , resulting in its continuous degradation [3]. Interference with NF- κ B activity, either by ectopic expression of a dominant-negative form of IKK β or a non-degradable I κ B α , super-repressor, causes cell cycle arrest and apoptosis in cell line models of ABC DLBCL. In addition, small molecule inhibitors that interfere with either the degradation of I κ B α , or the activation of IKK β are selectively toxic for ABC DLBCLs both in vitro and in the clinic [4, 7].

B cells activate NF- κ B transiently downstream of numerous receptors, including the B cell antigen receptor (BCR), CD40, the BAFF receptor and various Toll-like receptors (TLRs). The NF- κ B signaling addiction of ABC DLBCL raised the question if engagement of any of these upstream signaling pathways is present in ABC DLBCL. A loss-of-function RNA interference (RNAi) screen was performed on DLBCL cell lines to identify genes whose expression was essential for malignant cell proliferation and survival [8]. RNAi-mediated knockdown of IKK β expression was toxic to ABC, but not GCB, cell lines. The study further revealed an unappreciated role for the CARD11-BCL10-MALT1 (CBM) complex, a well-known upstream mediator of IKK activation and NF- κ B induction [9], as knockdown of each component was toxic to ABC DLBCL lines.

The CBM complex is critical to the activation of the classical NF- κ B pathway downstream of the B and T cell antigen receptors [9]. In B cells, BCR signaling activates PKC β , which in turn phosphorylates CARD11, triggering it to adopt an active conformation. Active CARD11 can translocate to the inner side of the plasma membrane where it associates with BCL10 and MALT1 to form an active CBM complex. CARD11 is central to ABC DLBCL pathogenesis as evidenced by activating mutations found in ~10% of ABC DLBCL tumors [10]. Recurrent mutations in the coiled-coil domain of CARD11 allow constitutive membrane association, CBM nucleation, and subsequent activation. Mutant forms of CARD11 form clusters that co-localize with MALT1 and IKK β , whereas wild-type CARD11 is generally diffuse throughout the cytoplasm. Furthermore, the level of constitutive NF- κ B activity directly correlates with the amount aggregation of found in CARD11 mutant-expressing cells, underscoring the pathogenic relevance of these mutations to ABC DLBCL. Silencing members of the CBM complex decreases NF- κ B activity and is toxic to ABC DLBCL [10].

Recently, another component of the CBM was defined by analysis of ABC DLBCLs, the linear ubiquitin chain assembly complex (LUBAC). The importance of LUBAC to ABC DLBCL biology was revealed by rare single nucleotide polymorphisms (SNPs) affecting a subunit of the linear ubiquitin chain assembly complex (LUBAC), RNF31. These SNPs were substantially enriched in ABC DLBCL (~8% prevalence) as compared to other subtypes of lymphoma and to the population at large (~1% prevalence) [11]. Linear ubiquitination of the IKK gamma (NEMO) subunit by LUBAC is required for IKK activation in response to several upstream inputs. LUBAC is physically associated with the CBM and knockdown of LUBAC components kills ABC DLBCL cells [11, 12]. Notably, the LUBAC SNPs augment linear ubiquitination of IKK, increase NF- κ B activity and enhance the survival of ABC DLBCL lines, suggesting that inheritance of these SNPs may predispose individuals to this cancer type [11].

Transcriptional Feedback

NF- κ B activation is frequently depicted as the terminus of multiple prosurvival signaling pathways [6]. However, in ABC DLBCL, activation of NF- κ B is better described as an amplification of upstream oncogenic signaling since it creates multiple feed forward and feed back signaling loops through transcriptional activation of target genes necessary for survival [13]. For instance, NF- κ B promotes the secretion of IL6 and/or IL10 in most ABC DLBCL [14]. These cytokines can interact with cytokine receptors expressed on the very same cells.

Downstream activation of JAK/STAT signaling further enhances NF- κ B activity as the STAT3 transcription factor can physically interact with NF- κ B, creating an autocrine feed forward loop that further enhances NF- κ B target gene expression. Indeed, ABC DLBCL tumors that activate JAK/STAT signaling have higher NF- κ B target gene expression, including the anti-apoptotic gene A1 (BCL2A1), as well as higher expression of glycolysis and proliferation genes, due in part to increased MYC expression.

Additionally, NF- κ B directly induces the expression of IRF4, an essential transcription factor for the survival of this lymphoma subtype [15, 16]. An RNAi screen demonstrated that both IRF4 and its DNA-binding partner SPIB are required for ABC DLBCL survival [15]. IRF4 and SPIB contribute to the expression of the critical CBM component CARD11 in ABC DLBCLs via a self-reinforcing positive feedback loop. IRF4 and SPIB also repress an otherwise toxic Type I interferon response in ABC DLBCLs that would normally be activated by MYD88 signaling. Thus, disrupting IRF4 activity is toxic to ABC DLBCLs due to the collapse of both pro-survival NF- κ B signaling and suicide by autocrine induction of interferon [15]. Finally, IRF4 has been found to have an additional binding partner in lymphocytes, BATF, which can influence the spectrum of genes targeted during activation and differentiation [17, 18]. IRF4 and BATF are required downstream of BCR signaling to promote transcriptional programs during GC formation [17]. The association of IRF4 with SPIB versus BATF generates heterodimeric transcription factors that control distinct and developmentally-related gene expression programs in DLBCL, and they can be used to identify a subset of ABC DLBCL patients (SPIB-high/BATF-low) with MYD88 L265P who may have a better clinical outcome [18]. Thus, oncogenic signaling downstream of the BCR

creates multiple self-reinforcing survival signals through the amplification of various NF- κ B transcriptional networks in ABC DLBCL.

BCR signaling

Human lymphomas, including DLBCL, retain expression of their BCR even though chromosomal translocations frequently disrupt the IgH locus [19]. The BCR is composed of pairs of heavy (IgH) and light (IgL) chains that form a surface-expressed antibody, coupled to a CD79A (Ig α) and CD79B (Ig β) heterodimer, which is required for plasma membrane expression, intracellular trafficking and signal transduction. IgH and IgL are composed of constant regions that are fused to antigen recognition variable regions, which are constructed from V_H, D_H and J_H gene segments for IgH, and V_L and J_L gene segments for IgL. In the case of IgH, naïve B cells express IgM and IgD constant regions, but can change to either IgG, IgA or IgE constant regions following class switch recombination (CSR) in the germinal center. ABC DLBCL cases predominantly express an IgM-BCR, while GCB DLBCL cases have typically switched to an IgG-BCR [20], although both tumor types are derived from cells that have experienced a germinal center reaction. This is due, in part, to mutations in the switch μ , region of the IgH gene in ABC DLBCL that interfere with its function to promote class switching from IgM to other isotypes [20]. These inactivation events occurs specifically on the productive (in-frame) IgH allele in ABC DLBCL tumors whereas the non-productive (out-of-frame) IgH alleles often have switched to another isotype [21]. Thus, there appears to be selective pressure for ABC DLBCL tumors to maintain expression of an IgM-BCR. This is possibly because IgM- and IgG-BCRs induce qualitatively distinct signaling cascades and, ultimately, transcriptional programs [22–25]. IgM-BCR signaling preferentially induces pro-survival and mitogenic signals, including NF- κ B, while IgG-BCR signaling favors plasma cell differentiation [25]. NF- κ B activation is the defining feature of ABC DLBCL and thus IgM-BCR signaling may be part and parcel of this transcriptional profile.

Antigen-dependent aggregation of the BCR rapidly induces phosphorylation of multiple downstream signaling effectors (reviewed in [26]; Figure 1A). The first biochemical step of BCR signaling is the phosphorylation of tandem tyrosines within immunoreceptor tyrosine-based activation motifs (ITAMs) present on both CD79A and CD79B by members of the Src-family kinases [27]. Dually phosphorylated ITAMs recruit and activate Syk kinase, which in turn autophosphorylates itself to create docking sites for additional effectors, including the adaptor BLNK and BTK [28]. SYK and SRC kinases can then activate Btk, which is simultaneously recruited to the plasma membrane by the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) by phosphoinositide 3-kinase (PI3-K) [29]. PI3-K is activated through BCR-dependent phosphorylation of CD19 by Src-family kinases. Once Btk is recruited and activated in the BCR signalosome, it phosphorylates phospholipase C γ (PLC γ), which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to the IP3 receptor on the endoplasmic reticulum, causing release of intracellular calcium stores, ultimately initiating an extracellular calcium flux and activation of many calcium-dependent signaling and transcriptional programs. The combination of increased intracellular calcium and DAG production activates protein kinase C beta (PKC β). PKC β

phosphorylates CARD11, thereby inducing a conformational change that nucleates CBM complex formation, triggering the classical NF- κ B pathway. BCR stimulation also initiates signaling through the ERK, MAPK and NF-AT pathways, which coordinate with NF- κ B to promote B cell survival, proliferation and differentiation [26].

BCR signaling in ABC DLBCL

Although CARD11 mutation represents a bona fide route to transformation in ABC DLBCL, the majority of ABC tumors do not have CBM mutations despite reliance on its presence, suggesting further upstream pathogenic dysfunction. The CBM complex is known to relay signals from the BCR to NF- κ B. The role of BCR signaling in ABC DLBCL was first suggested from an RNAi screen indicating that Btk was critical for tumor survival in ABC lines with wild type CARD11, but not to GCB lines or an ABC line with a CARD11 mutation [30]. Btk is primarily thought to function downstream of the BCR in B cells, but its expression is not confined to B cells, nor to BCR signaling [31], necessitating further evidence to tie ABC DLBCL survival to BCR signals. Targeted knockdown of BCR components, including immunoglobulin heavy and light chains, CD79A and CD79B, as well as key signaling effectors downstream of the BCR (Syk, Blnk, PLC γ 2, PKC β) is toxic to ABC DLBCL lines with wild type CARD11, but not GCB lines [30]. Likewise, pharmacological inhibition of Syk, Btk and PKC β selectively kills ABC DLBCL cell lines with wild type CARD11, confirming the importance of the BCR signaling pathway to the survival of ABC DLBCL cell lines.

Broadly speaking, there are two modes of signaling involving the BCR: antigen-independent, or “tonic”, signaling (Figure 1C), and antigen-dependent, or “active”, BCR signaling (Figure 1B). Tonic BCR signaling was defined by the observation that conditional ablation of surface BCR expression in mouse B cells results in the eventual loss of all naïve mature B cells [32, 33]. Tonic BCR signaling requires the ITAM portion of CD79A [33], but may not require the extracellular portions of IgM [34], suggesting that this mode of BCR signaling is antigen-independent. A constitutively active form of PI3-K was able to rescue the survival of mouse B cells in which the BCR was genetically ablated, suggesting a key and perhaps sole role for PI3-K in delivering survival signaling during tonic BCR signaling [35]. In contrast, active BCR signaling occurs subsequent to BCR aggregation, as described above. Unlike tonic BCR signaling, active BCR signaling engages many pathways and transcriptional networks, with CARD11-mediated activation of NF- κ B being characteristic of this mode of BCR signaling. Genetic or pharmacologic inhibition of BCR signaling in ABC DLBCL lines disrupts NF- κ B signaling, suggesting that BCR signaling in ABC DLBCL is more akin to active BCR signaling in normal B cells. By contrast, GCB DLBCL lines do not rely on NF- κ B for survival [3, 30], but a subset of these lines may rely on activation of PI3-K by tonic BCR signaling (see below) [36]. BCR signaling in ABC DLBCL also engages the PI3-K pathway [30], which may indirectly contribute to NF- κ B activity [37]. Microscopic examination of the BCR on the surface of ABC DLBCL cell lines and patient samples revealed a consistent pattern of BCR clustering [30] reminiscent of BCR clusters observed in antigen-stimulated B cells [38], whereas BCR clusters were not observed in GCB DLBCL lines. Taken together, these results suggest that the pathological

BCR signaling in ABC DLBCL resembles antigen-dependent active BCR signaling in normal B cells and thus has been termed “chronic active” BCR signaling.

Sequencing revealed recurrent mutations affecting the ITAM regions of CD79A and CD79B. CD79B mutations are substantially more prevalent (~20%) than CD79A mutations (~3%). The mutations in CD79B almost exclusively affect the membrane-proximal tyrosine of the ITAM motif, changing this amino acid to any other, while the CD79A mutations delete most of the ITAM region [30]. Despite the fact that these mutations target the motifs known to be required for signaling transduction by the BCR, the CD79A/B mutations in ABC DLBCL are not loss-of-function as they support chronic active BCR signaling [30]. However, unlike CARD11 mutants in DLBCL, CD79A/B mutations are not better at inducing NF- κ B activity than their wild-type counterparts, nor do the mutations enhance BCR clustering [30]. Work is ongoing to understand the molecular mechanisms by which these mutants are selected in ABC DLBCL, and how they contribute to the pathogenesis of the disease. One function of the ITAMs is to mediate receptor internalization, which eventually terminates BCR signaling [39]. CD79A/B mutations present in ABC DLBCL were found to promote increased expression of the BCR on the cell surface [30]. Recent cell biological investigations revealed that the membrane-proximal ITAM tyrosine of CD79B is the only ITAM tyrosine that is required for receptor endocytosis in a model system involving both the CD79B and CD79A cytoplasmic domains [40], potentially explaining why point mutations in ABC DLBCL only target this CD79B tyrosine. Nonetheless, the CD79A cytoplasmic domain is required for endocytosis of CD79B [40], providing insight into why the CD79A ITAM is deleted in some ABC DLBCLs. In addition their effect on BCR internalization, BCR signaling in the context of CD79B mutations is less able to activate Lyn kinase relative to signaling by receptors containing wild type CD79B [30]. While Lyn kinase plays a role, along with other Src-family kinases, in initiating BCR signaling, Lyn kinase activity can also dampen BCR signaling via phosphorylation of CD22 and recruitment of the phosphatase SHP-1, which dephosphorylates BCR ITAM tyrosines [41]. Thus, reduced LYN kinase activity in the context of CD79 mutations in ABC DLBCL may augment ongoing chronic active BCR signaling.

NF- κ B activation in ABC DLBCL can also be augmented by inactivation of *TNFAIP3*, which encodes A20, a negative regulator of IKK [42]. *TNFAIP3* is inactivated by several mechanisms, including deletion, point mutation and epigenetic silencing in ABC DLBCL and other lymphoma subtypes. The mechanisms by which A20 curtails NF- κ B activation are complex, but may include deubiquitination and steric hindrance of ubiquitin-mediated protein-protein interactions (reviewed in [43]). Since *TNFAIP3* is a direct transcriptional target of NF- κ B, inactivation of A20 is a frequent mechanism to promote constitutive NF- κ B activation in lymphoma.

The origins of chronic active BCR signaling

The resemblance of chronic active BCR signaling to antigen-stimulated active BCR signaling in normal B cells raises the possibility that an autoantigen drives BCR clustering and subsequent NF- κ B signaling in ABC DLBCL. The potential role of autoantigens in the pathogenesis of chronic lymphocytic leukemia (CLL) has been well studied and is

instructive in this regard. BCR signaling has long been suspected as a driver of pro-survival signaling in CLL cells, but it has been difficult to obtain direct evidence. Instead, investigators have studied the antigen recognition portions, or variable (V) regions, of IgH to discern the history of that B cell clone. The malignant cells in CLL frequently utilize a small subset of V_H gene segments out of the scores available in the B cell repertoire [44], suggesting a selective bias shaped by antigen. Moreover, CLL patients not only disproportionately use the same V_H gene segment, but unrelated patients can have nearly identical V region sequences, constructed from the same V_H D_H and J_H segments and paired with the same light chain variable regions as well. These so-called stereotyped receptors can account for over 30% of CLL cases [44], and indicate that CLL cells from different patients may recognize the same antigen. Studies have suggested that CLL cells can recognize a multitude of autoantigens, including proteins found in dying cells [45, 46]. More recently, it was shown that many CLL BCRs recognize a highly conserved epitope within the relatively invariant Framework 2 region of the heavy chain V region [47]. In this case, the BCR acts as its own antigen to induce active BCR signaling. Similar types of autoantigens may exist in the context of ABC DLBCL. Examination of non-GCB DLBCL V gene segments found a recurrent usage of certain V gene segments, implicating a selective process shaped by antigen. Prominent among these V gene segments was VH4-34, which is also frequently used in CLL [48]. Since chronic active BCR signaling occurs in cell line models of ABC DLBCL, at least some autoantigens must be expressed in vitro in the lymphoma culture, independent of a tumor microenvironment. One could therefore hypothesize that ABC DLBCL tumors recognize antigens similar to those used by CLL.

The hypothetical involvement of autoantigen signaling in ABC DLBCL raises the question of how the normal B cell progenitor of ABC DLBCL escapes immune tolerance mechanisms. Throughout the process of B cell development immature B cells must express a pre-BCR or BCR to survive, but ligation of this BCR by an autoantigen usually leads to cell death and deletion of the autoreactive clone. Some B cells expressing a self-reactive BCR may escape clonal deletion in the bone marrow to become mature B cells in the periphery. In order to prevent autoimmunity, these B cells are kept in an unresponsive state, known as anergy, which is characterized by reduced surface expression of the BCR and decreased cellular signaling [49]. One could imagine that ABC DLBCL tumors may arise from cells that have broken anergy and were able to enter the germinal center, where a given clone acquired additional oncogenic mutations through the process of somatic hypermutation (SHM) or class-switch recombination. The CD79 mutations in ABC DLBCL may provide one means to break anergy. As mentioned above, BCRs bearing these mutated CD79 subunits are defective in endocytosis and are retained on the plasma membrane, thereby potentially countering the down-modulation of surface BCR expression that characterizes anergic B cells. Secondly, high activity of Lyn kinase is a feature of anergic B cells in the mouse[50], and thus the attenuation of this kinase by the CD79 mutations in ABC DLBCL may contribute to the loss of anergy.

Cooperation of BCR and MYD88 signaling in ABC DLBCL

The BCR pathway and mutant CARD11 are not the sole sources of NF- κ B activity in ABC DLBCL. Toll-like receptor (TLR) signaling through the adapter MyD88 is a potent inducer

of NF- κ B activity in normal immune cells. An RNAi screen revealed that most ABC DLBCL lines are dependent on the expression of MyD88 and its associated signaling kinase, IRAK1 [51]. Additionally, high-throughput mRNA sequencing of DLBCL samples revealed recurrent mutations affecting the Toll/Interleukin-1 receptor (TIR) domain of MyD88 [51], which is necessary for homo- and heterodimeric interactions with upstream receptors, such as the TLRs [52]. Resequencing of *MYD88* in DLBCL clinical samples revealed that 39% of ABC DLBCL cases express a mutant isoform of MyD88, with the majority (29% of all cases) bearing a Leucine to Proline substitution at position 265 (L265P) [51]. Mutant MYD88 isoforms are potent activators of NF- κ B: knockdown of endogenous MyD88 in ABC DLBCL cells decreases NF- κ B activity while ectopic expression of the MyD88 mutants in heterologous cell systems induces NF- κ B [51]. In ABC DLBCL lines with MyD88 L265P, wild-type MyD88 cannot substitute for the mutant isoform, demonstrating the oncogenic nature of this MyD88 mutation.

Conceivably, mutation of *MYD88* could define a BCR-independent pathway to activate NF- κ B and promote ABC DLBCL cell survival. However, the L265P *MYD88* mutation co-occurs with *CD79A* and *CD79B* mutations in ABC DLBCL more often than expected by chance, suggesting cooperation rather than redundancy [51]. B cells recognize and respond to complex antigens via both BCR and TLR engagement, and the utilization of activation signals from multiple receptors may facilitate the B cell responses [53]. Moreover, BCR signaling can influence TLR localization and downstream signaling in normal [54] and anergic [55] B cells. ABC DLBCL tumors may have co-opted both pathways to produce the most potent pro-survival signaling. In ABC DLBCL lines expressing both mutant CD79B and MyD88 L265P, knockdown of MyD88 results in decreased signaling downstream of the BCR (RMY, LMS unpublished results), providing evidence that BCR and MYD88 signaling in ABC DLBCL are linked. In addition, recent evidence suggests that mutant isoforms of MyD88 can help break B cell anergy in mouse B cells, providing another potential way in which an autoreactive mature B cell can break tolerance and give rise to ABC DLBCL [56].

Tonic BCR signaling in lymphoma

Given the required role for tonic BCR signaling in normal B cells, it is not surprising that this survival mechanism is co-opted by some forms of B cell lymphoma. A prime example is Burkitt lymphoma, an aggressive malignancy derived from the rapidly proliferating germinal center centroblasts (reviewed in [57]). An RNAi screen revealed that many Burkitt lymphoma cell lines rely upon the BCR for survival, but the majority of these did not require CARD11 and were not dependent upon the NF- κ B pathway [58]. Rather PI3-K signaling is a hallmark of this lymphoma subtype, and this is dependent upon the BCR [58]. Indeed, a mouse model of Burkitt lymphoma has been created in which constitutive activation of the PI3-K pathway is coupled by overexpression of Myc, the defining oncogene of this cancer [59]. Cancer gene resequencing demonstrated that roughly 70% of Burkitt lymphomas acquire mutations affecting the transcription factor TCF3 (E2A) and/or its negative regulator ID3, leading to hyperactivity of TCF3 [58]. TCF3 transactivates immunoglobulin heavy and light chain genes by binding to conserved E-boxes in their enhancers [60]. Consequently, the TCF3/ID3 mutations in Burkitt lymphoma upregulate expression of the BCR on the cell surface, promoting tonic BCR signaling. In addition, TCF3 directly represses *PTPN6*, which

encodes SHP-1, a phosphatase that attenuates BCR signaling by dephosphorylating the BCR ITAM tyrosines. Hence, the genetic abnormalities of Burkitt lymphoma augment the survival of these malignant cells by promoting tonic BCR signaling.

In contrast to ABC DLBCL, GCB DLBCLs do not acquire highly recurrent mutations in the BCR signaling or NF- κ B pathways. Moreover, they are not killed by depletion of CARD11 [8] nor by inhibition of IKK [7], suggesting that they do not rely on NF- κ B and the active form of BCR signaling. However, a subset of GCB DLBCL lines are dependent upon Syk-mediated induction of the PI3-K pathway, suggesting the possibility of tonic BCR signaling [36]. The importance of the PI3-K pathway in GCB DLBCL is underscored by inactivation of PTEN, a negative regulator of PI3-K, in more than 50% of cases by a number of mechanisms, including deletion, mutation and amplification of the miR17-92 microRNA cluster that downregulates PTEN expression [16, 61]. Further work will be necessary to elucidate whether the BCR and/or other membrane proteins are required for Syk kinase and PI3-K activation in GCB DLBCL, and to develop methods to identify the subset of GCB DLBCL that rely upon this mechanism for survival.

BCR pathway inhibitors in ABC DLBCL

The knowledge gained from elucidating the signaling pathways in DLBCL has led to the development of precise therapeutic interventions. Numerous BCR pathway inhibitors have entered the clinic to treat B cell lymphomas in recent years, targeting kinases proximal to the BCR (e.g. Syk, Btk and PI3-K) as well as downstream targets such as MALT1 protease activity (reviewed in [62]). One of the most promising new agents is the Btk inhibitor ibrutinib, which is an irreversible inhibitor that forms a covalent bond with Cysteine 481 adjacent to the active site of Btk, lending it a high degree of specificity and potency. Ibrutinib has been approved for use in relapsed and refractory CLL and mantle cell lymphoma, and is currently in clinical trials for DLBCL. A phase 2 trial in relapsed and refractory DLBCL found that 40% of patients with ABC DLBCL had an objective response to ibrutinib, compared with only 5% of patients with GCB DLBCL, supporting the hypothesis that ABC but not GCB DLBCL relies upon chronic active BCR signaling [63].

Acquired resistance to BCR pathway inhibitors is a challenge that must be surmounted in order to realize the full potential of these drugs. Recent work has described the acquisition of ibrutinib bypass mutations affecting Btk and its direct target PLC γ 2 in CLL [64, 65] and MCL [66] patients. To circumvent these resistance mutations in lymphomas, one possibility is to use combinations of targeted therapeutic agents to attack the malignant cells on multiple fronts. One way to identify the most potent combinations is through a deep understanding of the wiring diagram that governs oncogenic signaling in a particular lymphoma type. For example, the drug lenalidomide was observed to kill ABC DLBCL cells by two distinct mechanisms, both involving its ability to decrease the expression of IRF4 and SPIB, two essential transcription factors that dictate much of the biology of this lymphoma subtype [15]. On one hand, IRF4/SPIB are required for expression of CARD11 and thus are essential for full NF- κ B pathway activation in BCR-dependent ABC DLBCL. On the other hand, IRF4/SPIB repress IRF7, which promotes type I interferon expression. As a result, lenalidomide induces type I interferon expression in ABC DLBCLs with

MyD88 L265P, which kills the tumor cells by autocrine signaling through the type I interferon receptor. The *IRF4* gene is also directly transactivated by NF- κ B factors, and thus ibrutinib can also down-modulate its expression. Consequently, the combination of lenalidomide and ibrutinib is highly synergistic in killing ABC DLBCL cells both *in vitro* and in xenograft models [15]. On the basis of these findings, a multicenter phase two trial of this combination together with chemotherapy has been initiated. A second general strategy to identify potent synergistic drug combinations is to utilize robotic technology to screen pairs of compounds over a wide range of different concentrations [67]. A high-throughput screen of ibrutinib in combination with a library of almost 500 anti-tumor agents with survival of ABC DLBCL cells as the endpoint uncovered synergies between ibrutinib and several PI3-K pathway inhibitors, the BCL2 family inhibitors navitoclax and ABT-199, and various conventional chemotherapeutic agents [67]. A similar screen using JQ-1, an inhibitor of BET-domain chromatin regulators yielded a surprising synergy between this molecule and inhibitors of oncogenic signaling in ABC DLBCL [68]. This result led to the unexpected observation that BET-domain proteins are required to sustain the oncogenic activity of IKK in ABC DLBCL, and suggest that drugs targeting these proteins may find a place in combination therapies of ABC DLBCL in the future. Given the large and growing array of agents that can inhibit oncogenic signaling in ABC DLBCL (and other cancers), the burden lies with researchers to nominate a small number of combinations for clinical evaluation that are predicted to have maximal synergy on a mechanistic and experimental basis.

Conclusion

Cancer cells utilize a variety of mechanisms to promote their own growth and survival, including subverting cellular signaling networks. Antigen-dependent signaling through the BCR is highly mitogenic, but is a transient and self-limiting response to a foreign antigen in normal B cells. In contrast, ABC DLBCL tumors use a chronic form of active BCR signaling to maintain NF- κ B-driven growth and survival signals that promote malignant growth. BCR pathway inhibitors, such as the BTK inhibitor ibrutinib, have proven to be effective in the clinic to combat ABC DLBCL and other BCR-dependent lymphomas, including CLL and MCL. That said, it is apparent that lymphomas are able to eventually circumvent these selective kinase inhibitors and combination therapies will be necessary to truly extinguish these malignant clones. The continued application of genomic and chemical genetic technologies should allow us to identify novel pathways and develop rational combination therapeutics, thereby fostering the era of precision genomic medicine in lymphoma treatment.

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A. Active BCR signaling

B. Chronic Active BCR signaling in ABC DLBCL

C. Tonic BCR signaling: Burkitt lymphoma and GCB DLBCL(?)

FIGURE 1.

Different types of BCR signaling. A) Antigen-stimulated untransformed B cells; B) Chronic Active BCR signaling in ABC DLBCL; C) Tonic BCR signaling in Burkitt lymphoma and GCB DLBCL.